

The UL144 gene product of human cytomegalovirus activates NF κ B via a TRAF6-dependent mechanism

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Molecular mimicry of cytokines and cytokine receptors is a strategy used by poxviruses and herpesviruses to modulate host immunity. The human cytomegalovirus (HCMV) UL144 gene, situated in the UL/b' region of the viral genome, has amino-acid sequence similarity to members of the tumour necrosis factor receptor superfamily. We report that UL144 is a potent activator of NF κ B-induced transcription in a TRAF6-dependent manner. This NF κ B activation enhances expression of the chemokine CCL22 through the NF κ B responsive elements found in its promoter. In contrast to the clinical HCMV isolates, extensively passaged laboratory strains lack the UL/b' region and hence do not encode UL144. Consistent with this, infection with viruses that carry UL/b' causes NF κ B activation and CCL22 expression, a phenotype that is not observed after infections with strains lacking the UL/b' region. Moreover, knockdown of UL144, TRAF6 or NF κ B by specific siRNA in infections with UL144-encoding HCMV prevents the activation of CCL22 expression normally observed after infection with UL/b' positive HCMV. Upregulation of CCL22, which attracts Th2 and regulatory T cells, may help HCMV evade immune surveillance.

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Introduction

Human cytomegalovirus (HCMV) is a member of the beta-herpesvirus family. It is species-specific and can cause severe disease in neonates and in immunosuppressed individuals such as allograft transplant recipients and patients with AIDS (Pass, 2001), although the exact disease mechanisms are unclear. The HCMV genome comprises 236 kb of dsDNA, one of the largest human virus, and many of its genes are nonessential for replication in fibroblasts (Dunn *et al*, 2003). The long 15 kb unique region UL/b' contains at least 19 open

reading frames (ORFs) that are found in HCMV clinical isolates but have been lost in extensively passaged laboratory strains and are dispensable for growth *in vitro* (Cha *et al*, 1996). The retention of these ORFs in HCMV clinical isolates together with recent evidence that clinical isolates, but not the attenuated laboratory strain AD169, can replicate in SCID mice into which human tissues were implanted suggest that the UL/b' region is required for viral infection *in vivo* (Wang *et al*, 2005). Consistent with this, some of the UL/b' region genes may be implicated in immune evasion, such as the chemokine homologues UL146 and UL147 (Hengel *et al*, 1998; Mocarski, 2002). The UL144 ORF is encoded in the UL/b' region and a candidate for a pathogenesis marker because of its sequence similarity to the herpes simplex virus entry mediator (HveA or HVEM) (Benedict *et al*, 1999) and other members of the tumour necrosis factor receptor superfamily (TNFRSF) (Locksley *et al*, 2001; Ware, 2003), which are critical for defence against viral pathogens such as HCMV (Quinnan *et al*, 1984).

Avoidance of the host immune system by viruses is commonplace (Alcami and Koszinowski, 2000; Tortorella *et al*, 2000). Some viruses, such as Adenovirus, HCMV and Epstein–Barr virus (EBV), prevent cell surface expression or colocalization of specific receptors. Mimicry of cytokines and cytokine receptors is a mechanism of immune evasion identified in large DNA viruses (poxviruses and herpesviruses) (McFadden and Murphy, 2000; Alcami, 2003). Poxviruses encode secreted decoy receptors for cytokines and chemokines that intercept immune communications. Poxvirus TNFR homologues have amino-acid sequence similarity to the extracellular TNF binding domain of the cellular counterparts but lack the transmembrane and cytoplasmic domains, and hence they are secreted and neutralize TNF activity. A homologue of CD30, another TNFRSF member, has also been identified in poxviruses. UL144 encoded by HCMV is the only TNFRSF member identified in herpesviruses. In contrast to the poxvirus secreted TNFR homologues, UL144 has a transmembrane domain and a short cytoplasmic tail, and it is a membrane-anchored glycoprotein retained intracellularly (Benedict *et al*, 1999). UL144 may act as a decoy receptor but no interaction with members of the TNF superfamily (TNFSF) has been identified so far. Recently, the ectodomain of UL144 has been shown to interact with B and T lymphocyte attenuator (BTLA), belonging to the Ig superfamily, and to inhibit T cell proliferation *in vitro* (Cheung *et al*, 2005).

Viruses also modulate the immune response by interfering with transcription and signalling pathways of the host cell. The V protein of SV5, for example, causes the degradation of signal transducers and activators of transcription 1 and prevents dsRNA signalling to the interferon (IFN) promoter in an IFN regulatory factor 3 (IRF-3) dependent manner (Poole *et al*, 2002). The poxviral protein N1L blocks IRF-3 signalling via the toll-like receptor (TLR) pathway (DiPerna *et al*, 2004).

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Viruses also target the NFκB pathway, which is shared by several proinflammatory cytokines, such as TNF and interleukin 1 (IL-1), and TLRs (Li and Verma, 2002; Dempsey *et al*, 2003; Bonizzi and Karin, 2004). TNFR-activated factor 6 (TRAF6) is a mediator of NFκB activation and signals through protein kinase cascades via activation of the TAK1 complex. TAK1 phosphorylates and activates the IκB kinase complex, which then phosphorylates the NFκB inhibitor IκB, targeting it for ubiquitination and subsequent proteasome-mediated degradation. NFκB is then freed to enter the nucleus where it can act as a transcription factor. Viruses may prevent or enhance NFκB activation. For example, poxvirus proteins N1L and K1L interfere with the IκB kinase complex (DiPerna *et al*, 2004; Shisler and Jin, 2004) and prevent NFκB activation whereas latent membrane protein 1 (LMP1) protein of EBV activates the TRAF-mediated NFκB pathway (Schultheiss *et al*, 2001; Nakayama *et al*, 2004).

Here, we report that the UL144 protein of HCMV upregulates NFκB-dependent transcription through a mechanism involving TRAF6. We also show that this UL144-induced NFκB activation is concomitant with an increased expression of CCL22 (macrophage derived chemokine, MDC), a chemokine implicated in the attraction of Th2 cells, which may reduce host Th1 immune responses and be advantageous for HCMV *in vivo*.

Results

UL144 does not bind to members of the human TNFSF

Amino-acid sequence similarity of UL144 to HVEM and other TNFSF members has been reported but no binding of the recombinant UL144 ectodomain fused to the Fc portion of IgG1 to TNF-related ligands was detected by ELISA (Benedict *et al*, 1999; Lurain *et al*, 1999). We investigated whether UL144 interacts with members of the TNFSF by surface plasmon resonance (SPR, BIAcore X). The extracellular domain of UL144 from the HCMV low-passage strain Toledo, lacking the transmembrane domain and short cytoplasmic tail, was expressed in the baculovirus system and purified by affinity chromatography. We were unable to detect specific binding of 100 nM human recombinant TNFSF 1, 2, 4, 5, 6, 8, 9, 10, 11, 12, 13, 13b, 14, 15 and 18 to purified UL144 by SPR (not shown). As a control, we observed binding of the ectromelia virus TNFR CrmD to TNF (not shown).

UL144 in isolation and viruses containing UL144 are capable of inducing NFκB

The possibility that UL144 may modulate intracellular signalling pathways involved in antiviral responses was investigated (Figure 1). UL144 from HCMV Toledo was expressed in human U373 cells, a cell line fully permissive for HCMV productive infection, and tested for its ability to repress or activate these signalling pathways. Figure 1B shows that UL144 has no effect on basal or activated levels of IFN-stimulated gene expression to the ISRE reporter. UL144 also had no effect on the IFN-β promoter, which contains an NFκB binding site and neither did it have any effect on activation of this promoter by dsRNA (Figure 1A) or the dsRNA-induced activation of IRF3 (Figure 1C). However, UL144 may activate NFκB in other promoters as the dsRNA responsive promoter requires concomitant activation of other transcription factors, such as IRF-3 (Figure 1A). Interestingly, we found that UL144

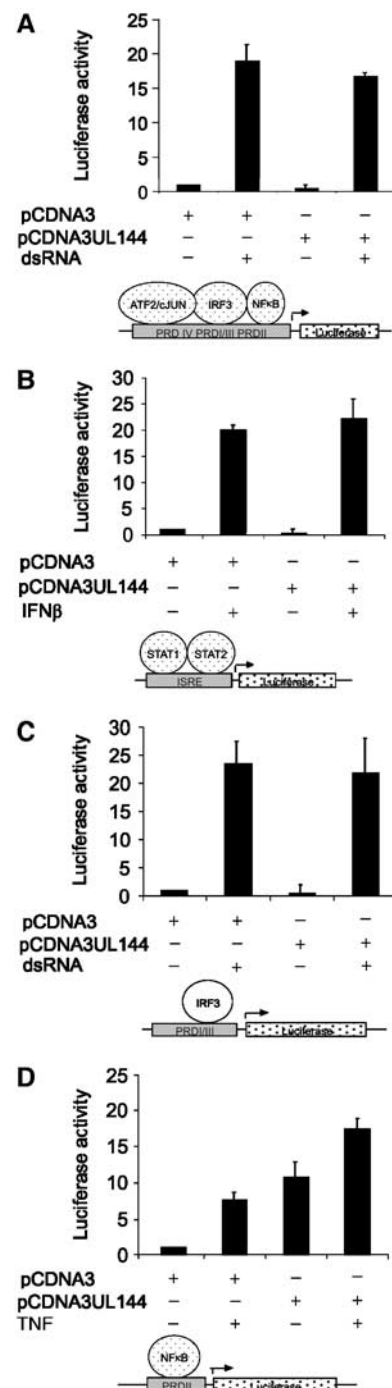


Figure 1 The UL144 protein of HCMV activates NFκB but not IRF3, IFN induction or response. Human U373 cells (A–D) were transfected with a luciferase reporter for IFN-β [pIFΔ(–125)lucifer] (A), type I IFN responsiveness [p(9–27ISRE)₄tkΔ(–39)lucifer] (B), IRF-3 activation [p(lexOP)2TKLuc and Lex-IRF3] (C) or NFκB activation [p(PRDII)₅tkΔ(–39)lucifer] (D) plus a reporter for β-galactosidase activity (pJATlac). Cells were also transfected with either a mammalian vector driving the overexpression of UL144 protein (pCDNA3UL144) or the control empty vector (pCDNA3). Forty-eight h post-transfection, cells were either mock-treated (–) or treated (+) with poly(I)–poly(C) (dsRNA) for 10 h (A, C), IFNα2a for 5 h (B) or TNF for 5 h (D). Luciferase activity was determined from cellular extracts and expression values calculated as the fold increase with respect to the level induced in cells transfected with pCDNA3 and reporter plasmid in the absence of stimulation. Experiments were repeated at least twice and mean ± s.d. from representative experiments (A–C duplicates and D, triplicate samples) is shown.

activated NF κ B-dependent activity of the PRDII promoter in the absence of inducers such as TNF. Additionally, UL144 and TNF had a cumulative effect indicating that the mechanism that UL144 uses to stimulate NF κ B may be separate from the TNF signalling pathway (Figure 1D). Specific activation of NF κ B was also observed in similar UL144 transfection with the PRDII promoter in experiments performed in Vero and 293 T cells (not shown).

To determine the ability of UL144 to activate NF κ B in the context of HCMV infection, viruses containing UL144 were compared with UL144 negative laboratory strains for their ability to activate an NF κ B responsive promoter PRDII in human U373 cells that support HCMV replication (Figure 2). At 48 h postinfection, when levels of infection were comparable for both UL/b' positive and negative HCMVs (Figure 2A) and the time at which UL144 is maximally expressed (Benedict *et al*, 1999), HCMV TB40E caused a potent activation of NF κ B but the UL144-deficient HCMV Towne did not (Figure 2B). Consistent with the results from UL144 expression alone, there was no difference in activation of the IFN-stimulated ISRE reporter (not shown). Furthermore, the inability of HCMV Towne, which does not contain the UL/b' region, to activate the PRDII NF κ B responsive promoter was complemented by transfection of pCDNA3UL144 (Figure 2C).

Consistent with the activation of NF κ B by UL144, we observed relocalization of the p65 subunit of NF κ B, both endogenous and transfected GFP p65, in cells transfected

with pCDNA3UL144 and not after transfection with control pCDNA3 (not shown). As predicted from the reporter assay, transfection of pCDNA3UL144 did not cause nuclear translocation of GFP IRF3 (not shown).

The mechanism of NF κ B activation by UL144 is TRAF6-dependent

NF κ B activation often involves one or more of the TNF signalling pathway components, such as TRAF2 and TRAF6. These proteins complex with cell surface proteins, such as TNFRs as well as those involved in release of NF κ B from the inhibitory protein I κ B (Li and Verma, 2002; Dempsey *et al*, 2003; Bonizzi and Karin, 2004). Initial immunofluorescence experiments in U373 cells showed no relocalization of TRAF2 following infection with HCMV strains containing or lacking UL144, or after UL144 transfection (not shown). Interestingly, infection with HCMV TB40E containing the UL144 gene caused TRAF6 (normally diffused and difficult to detect in the cytoplasm; Figure 3A), to accumulate in the perinuclear region (Figure 3B), whereas infection with the high passage HCMV AD169, lacking UL144, did not (Figure 3A). Perinuclear localization of TRAF6 has been reported previously and is believed to occur following activation, for example, CD40 ligation (Gentry *et al*, 2004).

We tested the possibility that UL144 and TRAF6 physically interacted *in vivo* by a number of techniques (Figure 4). Firstly, indirect immunofluorescence clearly showed that UL144 and TRAF6 co-localized in transfected cells (Figure 4A, right-hand panels). This was verified by using co-localization detection software (areas of co-localization shown in white in Figure 4B, left hand panel). We also analysed this co-localization by FRET analysis (Figure 4C). FRET occurs when two juxtaposed molecules reside 6 nm or less apart, and this analysis shows that red photobleaching of endogenous TRAF6 staining (Figure 4B, right-hand panel showing an absence of red staining) caused an increase in intensity of transfected UL144 green fluorescence (Figure 4C, left and right panels). Statistical analysis was consistent with

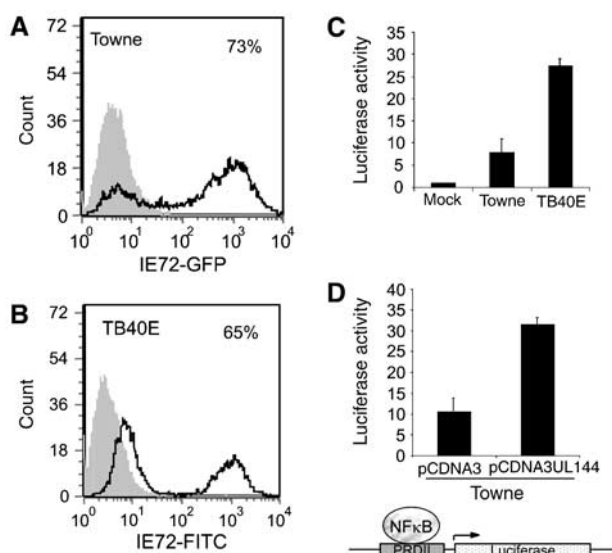


Figure 2 NF κ B activation with HCMV and UL144. Human U373 cells were infected with HCMV Towne (A) or TB40E (B) at an MOI of 1 PFU/cell. IE gene expression was quantified by FACS analysis of IE72-GFP (A) or with specific antibody to IE-72 (B). In (A), grey histograms represent uninfected controls and black histograms represent IE-GFP expression. In (B), grey histograms represent isotype stained controls and black histograms represent IE-72 positive cells. Numbers represent percentage of the population expressing IE genes. (C, D) U373 cells were transfected with the reporter construct for NF κ B activity [p(PRDI)₅tkΔ(-39)lucifer] and pJATlac (C, D) and pCDNA3 or pCDNA3UL144 (D) for 24 h before being mock-infected or infected with the indicated virus (moi 5 PFU/cell). Luciferase activity was determined 48-h postinfection from cellular extracts and the expression values calculated relative to the basal levels of luciferase activity in uninfected cells. Experiments were repeated at least twice and mean \pm s.d. from representative experiments (C and D, triplicate samples) is shown.

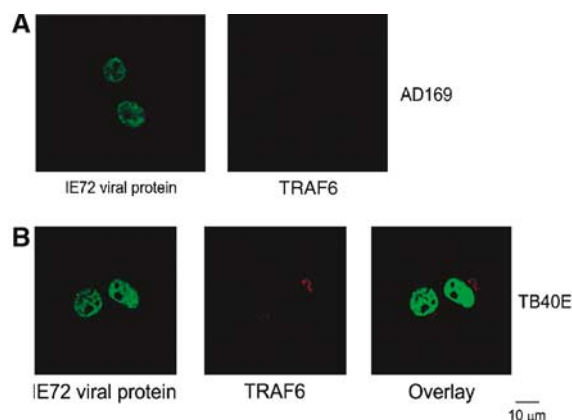


Figure 3 A UL144-containing HCMV, but not a virus lacking UL144, causes perinuclear accumulation of TRAF6. Human U373 cells were infected with HCMV AD169 (A) or TB40E (B). Infection was detected with FITC-conjugated monoclonal antibody specific for the HCMV immediate early protein IE72 (green, E13 antibody) and TRAF6 with rabbit polyclonal antibody followed by anti-rabbit TRITC (red).

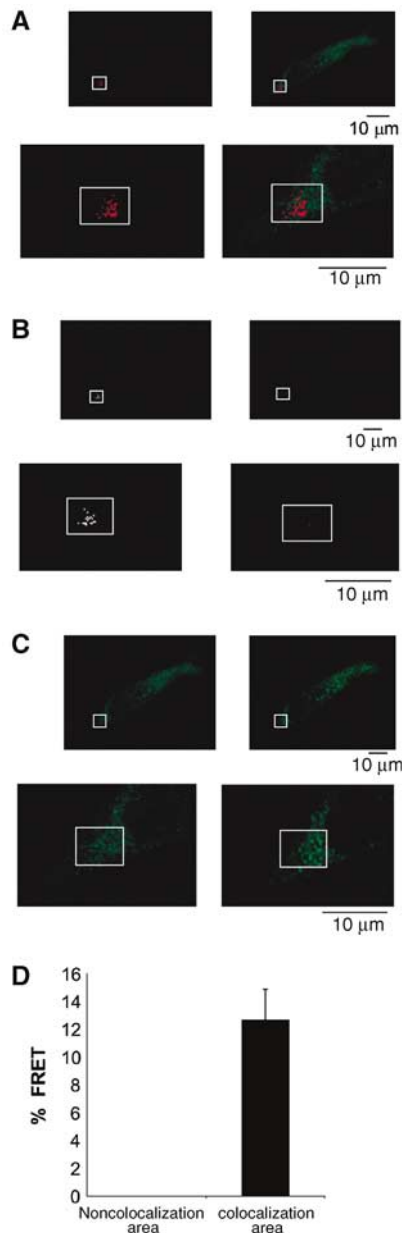


Figure 4 UL144 interacts directly with TRAF6. Human U373 cells transfected with pCDNA3UL144 were fixed and permeabilized 48 h post-transfection and probed with FITC conjugated anti-T7 (green) and polyclonal anti-TRAF6 followed by anti-rabbit TRITC (red). Cells were viewed by confocal microscopy either directly (pre-bleach) (A and left-hand panel of B) or with the inclusion of FRET (postbleach) (B, right-hand panel and C). In each case, the area of colocalization/photobleaching is shown magnified in the bottom panels. FRET images were analysed using Image J and the percentage of FRET analysis was plotted (D).

molecules of UL144 and TRAF6 being in very close proximity (Figure 4D). Taken together these results indicated that UL144 interacts with TRAF6 in the perinuclear region of the cell. Moreover, UL144 was able to complex with TRAF6 as determined by immunoprecipitation followed by Western blot analysis in UL144-transfected U373 cells (Figure 5) and 293 T cells (not shown). Note the specific co-precipitation of TRAF6 with antibodies to T7-tagged UL144 and not control IgG, from cell extracts containing comparable amounts of TRAF6.

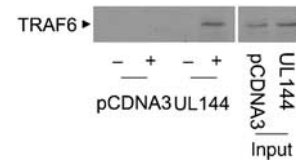


Figure 5 HCMV UL144 complexes with TRAF6. Human U373 cells were transfected with pCDNA3 or pCDNA3UL144 as indicated and immunoprecipitated with an irrelevant isotypic antibody (–) or anti-T7 tag (+) and analysed by SDS–PAGE and Western blotting using antisera specific for TRAF6. The presence of TRAF6 in cells transfected with pCDNA3 or pCDNAUL144 is shown (input). Molecular mass markers in kDa are indicated.

To test the functional significance of the interaction between TRAF6 and UL144, TRAF6 as well as TRAF2 expression were knocked down in U373 cells by siRNAs (Figure 6A). The amount of TRAF6 and TRAF2 proteins were completely abrogated after 2 days, whereas treatment with control siRNAs did not reduce either TRAF6 or TRAF2 levels. Figure 6B shows that UL144 was unable to activate an NFκB-dependent promoter (PRDII) after TRAF6 knockdown, but could activate it after transfection of cells with control or TRAF2 siRNA. These findings indicated that the mechanism by which UL144 activates the NFκB promoter is TRAF6-dependent, but not TRAF2-dependent. Furthermore, we also found that cells infected with HCMV TB40E expressing UL144 were unable to activate the NFκB responsive reporter in the presence of TRAF6 siRNA (Figure 6C), confirming that TRAF6 is involved in UL144-mediated activation of NFκB in the context of virus infection.

UL144-mediated activation of NFκB causes upregulation of the chemokine CCL22

Since the UL144 gene is contained within a region of the HCMV genome that is dispensable for virus replication in tissue culture, UL144 may play a role in the regulation of host genes involved in immune evasion. To test this, cytokine protein array analysis that included 120 cytokines and cytokine receptors was carried out on media from human foreskin fibroblast (HFF) cells infected with HCMV TB40E, containing UL144, or Towne, which lacks UL144. We found that several cytokines were affected differently by HCMV TB40E and Towne. Cytokines downregulated by HCMV TB40E included IGF BP-1 (insulin-like growth factor binding protein 1) (five-fold decrease); IL-4 (four-fold decrease); CXCL7 (neutrophil activating peptide 2, NAP2) (four-fold decrease); CCL5 (regulated on activation, normal T-cell expressed and secreted, RANTES) (three-fold decrease) and LTα (lymphotoxin α) (three-fold decrease). Cytokines upregulated included GDNF (glial cell line-derived neurotrophic factor) (three-fold increase); IL-1β (three-fold increase); IL-2 (five-fold increase); LIGHT (lymphotoxin-inducible expression competes with herpes simplex virus glycoprotein D for HVEM T-lymphocyte receptor) (four-fold increase) and CCL22 (seven-fold increase).

Since the chemokine CCL22 was the cytokine substantially upregulated, we explored this further. Figure 7 shows that supernatants from human U373 cells infected with UL144-expressing HCMV TB40E and Toledo upregulated CCL22 expression as determined by ELISA. Moreover, this increase in CCL22 expression was recapitulated in UL144 transfected cells. No such increase in CCL22 expression was observed in

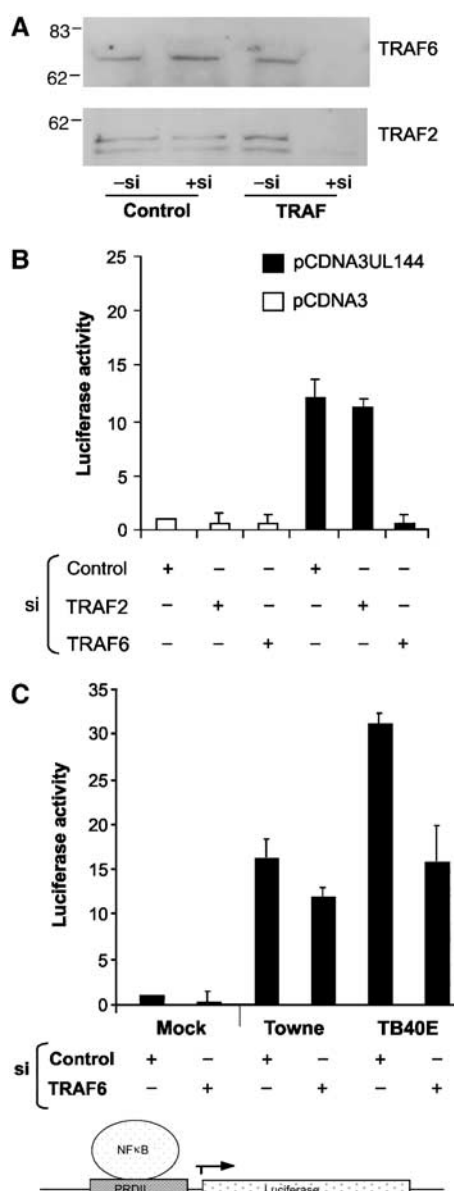


Figure 6 UL144 and HCMV activation of NFκB is dependent upon TRAF6 but not TRAF2. (A) TRAF6 and TRAF2 expression is abrogated by specific siRNAs. Human U373 cells were transfected in the absence (—si) or presence (+ si) of siRNAs specific for TRAF6 alone, TRAF2 alone or control siRNA as indicated. Cells were harvested 48 h post-transfection and analysed by Western blotting with antisera specific for TRAF6 and TRAF2. (B) Human U373 cells were transfected with a reporter for NFκB activation [p(PRDI) 5tkΔ(–39)lucifer] and co-transfected with pCDNA3 or pCDNA3UL144 and control, TRAF2 or TRAF6 siRNAs as indicated, as well as pJATlac. (C) Alternatively, U373 cells were transfected as in (B) but without pCDNA3 or pCDNA3UL144 and instead were mock-infected or infected with the indicated virus for 24 h post-transfection with control or TRAF6 siRNAs as indicated. Luciferase activity was determined 48 h later from cellular extracts and the expression values calculated relative to the basal levels of luciferase activity in cells uninfected and transfected with control siRNA. Experiments were repeated at least twice and mean±s.d. from representative experiments (B duplicate and C, triplicate samples) is shown.

cells infected with HCMV Towne or control transfected cells. Interestingly, HCMV Towne was able to induce CCL22 when cells were transfected with pCDNA3UL144 (Figure 7). Consistent with our observation that UL144 may mediate

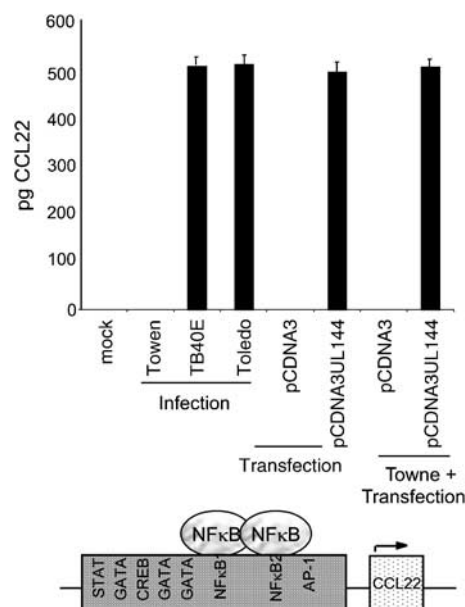


Figure 7 UL144 and HCMV containing UL144, but not laboratory strains, induce CCL22 expression. Human U373 cells were mock-infected or infected with the indicated viruses, or transfected with pCDNA3 or pCDNA3UL144 as indicated. Supernatants were collected at 48 h post-infection/transfection and processed for ELISA analysis. Results are plotted as pico grams of CCL22 detected in supernatants. A schematic representation of the transcriptional elements of the CCL22 promoter is shown. The experiment was carried out twice and mean±s.d. from a representative experiment of triplicate samples are shown.

the activation of specific NFκB-dependent promoters, the CCL22 promoter contains two NFκB binding sites (Nakayama *et al*, 2004). This chemokine is chemoattractant for Th2 and T regulatory cells, and therefore UL144 may evade the immune system by enhancing the Th2 response and subverting the Th1 response.

UL144 expressed from HCMV induces CCL22 upregulation through a TRAF6- and NFκB-dependent mechanism

The results reported above suggested that the upregulation of the chemokine CCL22 in cells infected with UL144-containing HCMV was mediated by UL144 expression; transfection of UL144 was sufficient to induce the activation of NFκB-dependent promoters through TRAF6 and upregulation of CCL22 expression, and this effect was not observed in cells infected with laboratory strains of HCMV lacking UL144. However, the UL/b' region present in the HCMV strains tested here encode up to 18 other ORFs and these other proteins may also activate NFκB. The rescue of CCL22 expression in cells infected with laboratory strains after UL144 transfection suggested that UL144 alone was responsible for CCL22 upregulation. We confirmed this by blocking UL144 expression in HCMV TB40E-infected U373 cells with siRNAs. Since UL144 is highly variable among HCMV clinical isolates (Lurain *et al*, 1999), the sequences of the UL144 siRNAs were designed to specifically inhibit the expression of UL144 encoded by either TB40E or Toledo. The effectiveness and specificity of these siRNAs to inhibit UL144 expression was monitored (Figure 8A). Densitometric analysis showed 85% inhibition of UL144 expression with TB40E-specific siRNA and 77% inhibition with Toledo-specific siRNA.

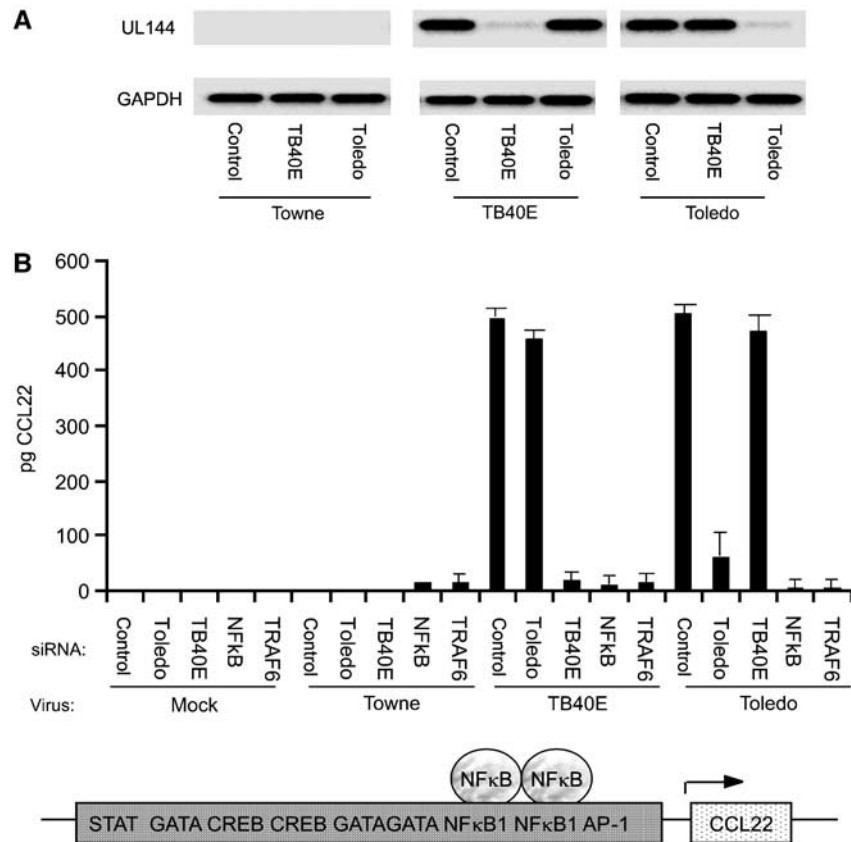


Figure 8 The upregulation of CCL22 during infection with HCMV TB40E or Toledo requires UL144, TRAF6 and NFκB. Human U373 cells were transfected with control siRNA or siRNA specific for UL144 from HCMV TB40E, UL144 from HCMV Toledo, TRAF6 or NFκB, as indicated. At 24 h later, mock-infected or infected with the indicated viruses. The efficiency of the siRNAs to prevent expression of UL144 was shown by RT-PCR (A). Supernatants were collected 48 h post-transfection and the amount of CCL22 determined by ELISA (B). The experiment was carried out twice and mean \pm s.d. from a representative experiment of triplicate samples are shown.

Figure 8B shows as expected that CCL22 upregulation was only observed in infections with HCMV TB40E and Toledo, and not in uninfected cells or cells infected with Towne. UL144-specific siRNAs specifically inhibited CCL22 upregulation by HCMV TB40E or Toledo demonstrating that UL144 is the only protein encoded by these viruses causing this effect. Moreover, the inhibition of CCL22 expression in HCMV TB40E infections by siRNA specific for UL144 from TB40E but not by siRNA specific for UL144 from the Toledo, and *vice versa*, further demonstrated the specificity of the siRNA inhibitory effect. The inhibition of CCL22 expression by TRAF6 or NFκB siRNAs confirmed that these cellular factors are also required for the UL144-induced CCL22 upregulation observed in infections with UL144-expressing HCMV (Figure 8B). Similarly, the CCL22 upregulation observed after infection of human fibroblast HFF cells infected with HCMV TB40E and Toledo was also inhibited by siRNA specific for UL144, TRAF6 and NFκB (not shown).

The LMP1 protein encoded by EBV also activates NFκB and induces CCL22 expression, and the NFκB sites in the CCL22 promoter are important for this upregulation of CCL22 transcription by LMP1 (Nakayama *et al*, 2004). However, UL144 may not work through the same mechanism. To address the dependence of the CCL22 promoter on NFκB sites in the regulation by UL144, a series of luciferase constructs driven by the CCL22 promoter and mutants thereof were tested (Nakayama *et al*, 2004). Figure 9A shows that removal of the NFκB sites specifically prevented UL144-mediated induc-

tion of the CCL22 promoter whereas deletion of the AP1 site had no effect.

The activation of the alternative pathway of NFκB activation by UL144 was addressed. Figure 9B shows that transfection of U373 cells with pCDNA3UL144 caused the specific degradation of NFκB2/p100 and the generation of the truncated p52 product, an event characteristic of the alternative pathway of NFκB activation (Bonizzi and Karin, 2004).

Discussion

The large coding capacity of the genome of herpesviruses and poxviruses enables the acquisition of a variety of genes that modulate the host immune response. Some of these immune evasion genes mimic cytokine and cytokine receptors involved in immune regulation (McFadden and Murphy, 2000; Alcami, 2003). Secreted decoy receptors for cytokines have been identified almost exclusively in poxviruses and only three examples have been described in herpesviruses (Alcami, 2003): a granulocyte-macrophage colony stimulating factor binding protein encoded by EBV and three chemokine binding proteins encoded by murine gammaherpesvirus 68, alphaherpesviruses and HCMV (Alcami, 2003; Bryant *et al*, 2003; Wang *et al*, 2004). These herpesvirus decoy receptors lack amino-acid sequence similarity to their cellular counterparts. By contrast, the UL144 protein encoded by HCMV is a homologue of HVEM and other TNFRSF members, and is anchored into cell membranes.

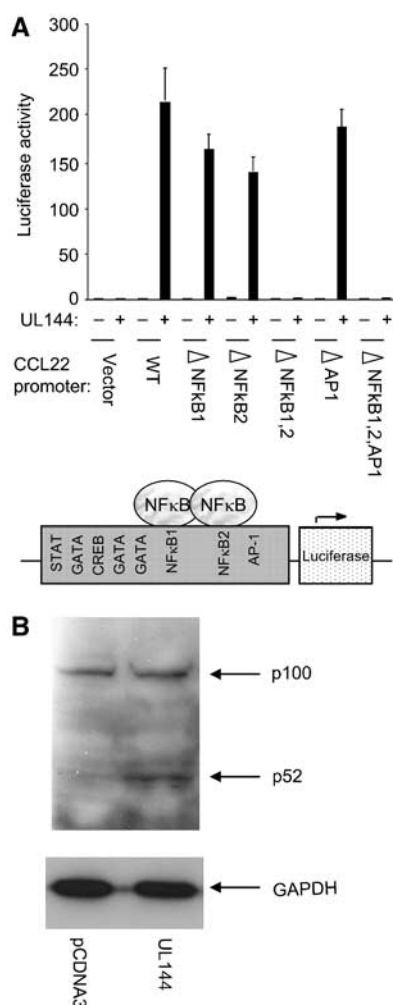


Figure 9 UL144-mediated upregulation of CCL22 is dependent upon the NF κ B site of the promoter and causes breakdown of NF κ B2/p100 into p52. Human U373 cells were transfected with pCDNA3 (–) or pCDNA3UL144 (+), the reporter plasmid pJATlac, and luciferase constructs driven by the wild-type CCL22 promoter (WT pGL3-CCL22, –722/–11) or with deletions (pGL3-CCL22-ΔNF κ B1, pGL3-CCL22-ΔNF κ B2, pGL3-CCL22-ΔNF κ B1 and 2, pGL3-CCL22-ΔAP1, pGL3-CCL22-ΔNF κ B1 and 2 and AP1) or empty vector pGL3 (vector) as indicated. At 48 h post-transfection, luciferase activity was determined from cellular extracts and the expression values calculated relative to the basal levels of luciferase activity in cells transfected with control vector pGL3. The experiments were carried out twice and mean \pm s.d. from a representative experiment of duplicate samples is shown (A). Alternatively, lysates were analysed by SDS-PAGE and Western blotting with antisera specific for p100/p52 or GAPDH (control for loading) (B).

The presence in UL144 of cysteine-rich domains (CRDs) characteristic of TNFRSF members suggests that UL144 may interact with TNFRSF members, but previous studies have failed to identify a ligand partner for UL144 by ELISA (Benedict *et al*, 1999). Here, we have extended those studies to other TNFRSF members and used a different and highly sensitive methodology (SPR) to screen for potential ligands, but we have failed to detect any specific interaction of TNFRSF members with UL144. The fact the UL144 lacks CRD3, which has been implicated in binding of TNFR to LT α , led to the suggestion that UL144 may not possess a ligand binding domain (Benedict *et al*, 1999). Recently, UL144 has been shown to interact with the Ig superfamily member BTLA, mimicking the interaction of HVEM with BTLA, and to inhibit

T-cell proliferation *in vitro* (Cheung *et al*, 2005). HVEM also interacts with LIGHT but no interaction of UL144 with LIGHT has been detected (this report) (Benedict *et al*, 1999).

In contrast to the decoy receptor function of the other viral cytokine receptors, the recent demonstration that UL144 interacts with BTLA suggests that UL144 mimics the inhibitory co-signalling function of HVEM and may modulate T-cell responses (Cheung *et al*, 2005). Here, we report a different and complementary function of UL144, which takes place within the infected cell: the activation of NF κ B signalling. This activation was demonstrated by expression of UL144 in cells and, most important, during the infection with UL144-containing HCMV. Altogether, several lines of evidence suggest that UL144 is the only gene responsible for the observed activation of NF κ B in HCMV infections: (i) this NF κ B activation does not occur in infections with HCMV laboratory strains lacking UL144; (ii) expression of UL144 in cells infected with UL144-deficient viruses induces NF κ B activation; and (iii) the HCMV strain-specific inhibition of UL144 expression with siRNA prevented CCL22 upregulation, which is dependent on NF κ B activation. The induction of the alternative pathway of NF κ B activation by UL144, causing the degradation of NF κ B2/p100 into p52 (Bonizzi and Karin, 2004), was also demonstrated in transfected cells.

The mechanism by which UL144 activates NF κ B is TRAF6-dependent and UL144 appears to achieve this by sequestering TRAF6 in the perinuclear region. Our results show that TRAF6 and UL144 are part of a larger complex and that they are in close proximity, as indicated by co-precipitation and co-localization analyses. However, we do not know whether UL144 and TRAF6 interact directly and the possibility that this interaction is mediated by other proteins has yet to be determined. The short cytoplasmic tail of UL144 has a YXXZ motif (Z is a hydrophobic amino acid) that is important in sorting of transmembrane proteins, and mutations of the tyrosine residue causes a higher expression of UL144 at the cell surface (Benedict *et al*, 1999). However, the cytoplasmic tail of UL144 lacks the motifs implicated in interactions with TRAFs. The inhibition of UL144-induced CCL22 upregulation by siRNA specific for TRAF6 or NF κ B establishes the requirement of these cellular factors for this function of UL144 within HCMV-infected cells.

The high amino-acid sequence variability in the UL144 protein encoded by HCMV clinical isolates is relevant considering the high conservation of other proteins, and has classified the isolates in three major groups (Lurain *et al*, 1999). Interestingly, all the amino-acid variability is found in the signal peptide and ectodomain of UL144, with 16–21% of amino-acid substitutions, mostly nonconservative, between isolates belonging to group 1 and groups 2/3. The finding that the transmembrane and cytoplasmic domains of UL144 are identical among 45 clinical isolates, with the exception of a R161K substitution in four isolates, is striking and strongly suggests that these domains are important for the correct function of UL144. The sequence of UL144 from the two UL144-containing viruses tested in our studies belong to the group 1 (Toledo) and group 3 (TB40E) and are highly divergent in their ectodomain (Dolan *et al*, 2004), with a single L151F substitution in the transmembrane domain of the UL144 from TB40E. Our finding that both these UL144 variants are able to induce NF κ B activation, transduce signalling through TRAF6 and upregulate CCL22 production, either

after UL144 transfection or upon virus infection, would be consistent with the conserved regions being involved in this function of UL144.

Viral proteins may evade cell signalling immune system pathways (Goodbourn *et al*, 2000; Andrejeva *et al*, 2002; Poole *et al*, 2002; Iqbal *et al*, 2004; Nakayama *et al*, 2004). Interestingly, like HCMV UL144, the LMP1 protein of EBV also upregulates NF κ B but through a different mechanism. LMP1 has six transmembrane segments and is unrelated to members of the TNFRSF, but its long cytoplasmic domain resembles that of TNFRSF members and acts as a docking site for recruiting and activating signalling proteins including TRAFs (Eliopoulos and Young, 2001; Schultheiss *et al*, 2001; Li and Chang, 2003). The HVEM homologue UL144 lacks a long cytoplasmic tail, but UL144 appears to act like LMP1 as a constitutive activator of NF κ B and also engages TRAF6 (Schultheiss *et al*, 2001). Thus, different human herpesviruses, EBV and HCMV, have evolved unrelated proteins to subvert the NF κ B pathway for their benefit.

The transcription factor NF κ B is critical for mounting both innate and adaptive immune responses to infection through induction of immune-related genes (Li and Verma, 2002). In addition, NF κ B activation upon HCMV infection facilitates viral immediate-early gene transcription and protein expression at late times of the HCMV replication cycle (Sambucetti *et al*, 1989; Yurochko *et al*, 1995). Recent evidence shows that NF κ B activation is not required for HCMV replication in fibroblasts but NF κ B signalling is important for the production and efficiency of antiviral factors such as TNF and IFN (Benedict *et al*, 2004; Eickhoff and Cotten, 2005). HCMV establishes latency in cells of the myeloid lineage and, although NF κ B may be involved in virus neutralization, NF κ B's exact role in virus reactivation and pathogenesis *in vivo* is not fully understood (Jarvis and Nelson, 2002). HCMV prevents external signalling to the cell by reducing cell surface expression of the 55-kDa TNFRI, a receptor that activates several signal transduction pathways including NF κ B (Baillie *et al*, 2003). At the same time, we show that HCMV triggers NF κ B signalling by expression of UL144, allowing the virus to modulate this pathway for its own benefit.

The biological relevance of the cellular genes affected by UL144-induced NF κ B activation for HCMV replication is of great interest. Here, we show that one end result of UL144-mediated NF κ B activation is the expression of CCL22, a chemoattractant for Th2 cells and regulatory T cells via CCR4 (Yoshie *et al*, 2001). Interestingly, this also parallels EBV since LMP1 triggers CCL22 expression (Nakayama *et al*, 2004). We propose that the upregulation of CCL22 will induce recruitment of Th2 cells that may unbalance an efficient antiviral Th1 response and, in addition, may enhance the migration of T regulatory cells with immunosuppressive properties to sites of infection. This would be complemented by the proposed additional function of UL144 as an inhibitor of T-cell activation through interaction with BTLA (Cheung *et al*, 2005) and by the ability of the chemokine receptor homologue US28 and the secreted chemokine binding protein UL21.5 encoded by HCMV to sequester CCL5, a chemokine that attracts Th1 cells and cytotoxic T cells via CCR5 (Bodaghi *et al*, 1998; Yoshie *et al*, 2001; Wang *et al*, 2004). These mechanisms would allow the virus to evade the host immune system. The results reported here establish a new function of UL144 that is demonstrated in the context of HCMV infection

and consists of the subversion of the NF κ B signalling pathway leading to upregulation of cytokines that may be advantageous for the virus.

Materials and methods

Cells and viruses

HFFs, U373 cells, Vero cells and 293 T cells were maintained in Eagle's minimal essential medium containing 10% fetal calf serum. Infections with HCMV AD169, TB40E and Toledo have been described (Murphy *et al*, 2002; Baillie *et al*, 2003). The HCMV Towne used in these studies was CR(IE1-GFP), a recombinant that does not express UL144 and encodes a fusion of IE1 to GFP, a kind gift of R Greaves (Imperial College School of Medicine, London) (Murphy *et al*, 2002). Insect Sf9 and Hi5 cells were used for expression of recombinant proteins.

Plasmids

The reporter plasmid with the firefly luciferase gene under the control of the human IFN- β promoter, pIFN(-125)lucifer, the synthetic PRD multimer reporters p(PRDI/III)₅tkA(-39)lucifer and p(PRDI)₅tkA(-39)lucifer, the IFN-responsive ISRE reporter p(9-27ISRE)₄tk(-39)lucifer and pEF-mlexA.IRF3, and the constitutive β -galactosidase reporter plasmid pJATlac were provided by S Goodbourn (St George's Hospital Medical School, University of London, UK) (Visvanathan and Goodbourn, 1989; Masson *et al*, 1992; King and Goodbourn, 1994, 1998; Poole *et al*, 2002). Plasmids containing wild-type CCL22 promoter (WT pGL3-CCL22, -722/-11) or with deletions (pGL3-CCL22- Δ NF κ B1, pGL3-CCL22- Δ NF κ B2, pGL3-CCL22- Δ NF κ B1 and 2, pGL3-CCL22- Δ AP1, pGL3-CCL22- Δ NF κ B1 and 2 and AP1) and the empty vector pGL3 were kind gifts of Dr O Yoshie (Kinki University School of Medicine, Osaka, Japan) (Nakayama *et al*, 2004). Expression of UL144 in mammalian cells was achieved by PCR of the UL144 gene from HCMV Toledo with primers 5'-CTAAGCCTGTAGGAGGCATG-3' (*HindIII*) and 5'-ATTGCTAGCCAGGGTCCGGTA-3' (*NheI*) (C-terminal tag) or 5'-CACGGGATCCAGTACTAAAGTGTGT-3' (*BamHI*) and 5'-ATGCGCGCCGTACAGGGTCCGGTA-3' (*NotI*) (N-terminal tag) and subcloning into pCDNA3 (Invitrogen) engineered to contain an N-terminal CD33 signal sequence followed by a T7 tag or a C-terminal T7 tag, provided by B Aguado (Centro de Biología Molecular, Madrid, Spain). Plasmids expressing UL144 with N- or C-terminal T7 tags were named pCDNA3UL144-T7N or pCDNA3UL144-T7C, respectively. Unless otherwise stated, both of these constructs were used in the experiments and the tag did not affect any of the UL144 functions tested. GFPp65 was a kind gift from W Greene (Gladstone Institute of Virology and Immunology, University of California, San Francisco) and GFP-IRF3 from S Goodbourn. T7 tagged WT-1 plasmid, pCDNA3WT1, was a kind gift of Dr Hastie (HGR, MRC, UK). The pBAC-1 plasmid (Novagen) expressing C-terminal his-tagged UL144 from HCMV Toledo in the baculovirus system was generated by PCR of the UL144 gene encoding the extracellular domain and lacking the transmembrane domain with primers: 5'-AAGGATCCCATGAAGCCTCTGATAATG-3' (*BamHI*) and 5'-TGCGGCCGACAGACAACCAGGCTAGAGT-3' (*NotI*). The DNA sequence of the constructs was confirmed.

Gene expression and protein analysis

For reporter gene assays, plasmids were transfected with Lipofectamine 2000 using the standard protocol except that following seeding overnight, cells were incubated for 5 h in OPTIMEM (Invitrogen) before transfection, allowing transfection of up to 80% of cells as tested by immunofluorescence. Lysates were prepared and analysed for luciferase and β -galactosidase activity (King and Goodbourn, 1994). For immunoprecipitations, pCDNA3UL144-T7N or pCDNA3 transfected cells were lysed in EBC buffer (50 mM Tris/Cl pH 8.0, 200 mM NaCl, 200 μ M Na₃VO₄, 10 mM NaF, 0.5% (v/v) NP40 and 50 μ M PMSF; supplemented with 40 μ g/ml BSA) and cellular debris removed by centrifugation. Lysates were precleared by addition of control Igs and protein A-Sepharose beads. For UL144 immunoprecipitation, cell lysates were incubated with anti-T7 antibody (Novagen) or isotype matched control antibodies followed by protein A-Sepharose:protein G-Sepharose (1:1) and washed with NETN (20 mM Tris/Cl pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% (v/v) NP40). Immunoprecipitates were resuspended in Laemmli's

buffer and proteins separated by SDS-PAGE. Proteins were then transferred to Hybond-C (Amersham Biosciences) and Western blotted with an anti-TRAF6 antibody (Abcam). Lysates from siRNA-transfected cells were Western blotted with anti-TRAF6 or anti-TRAF2 antibodies (Abcam). Rabbit polyclonal antibodies to NFκB2 p100 or GAPDH were from Cell Signalling or Abcam, respectively.

Design and transfection of siRNA

Removal of TRAF6 or UL144 by siRNA was achieved using three custom designed siRNA primers (Dharmacon) with the following sequences:

- (1) 5'-AATCAGAGCAAGTGATAATCA-3', 5'-AACGATCATGAACCTCTGAAG-3' and 5'-AACTGATTTGCACAAATAATA-3' for TRAF6.
- (2) 5'-AAAGATGAAGAGGGACAACCA-3', 5'-AACGGACAACGACGTATAATT-3' and 5'-AATTGTAACTCAGTGTCACCT-3' for UL144 from HCMV TB40E.
- (3) 5'-AATGCTCATCTGCTTTGCTGT-3', 5'-AAAGAGTTACTAAAGTATGCA-3' and 5'-ATAATACCGTATGCGCACCT-3' for UL144 from HCMV Toledo.

The siRNA for NFκB1 p105, NFκB p100 and TRAF2 were purchased from Qiagen. HFF or U373 cells in six-well plates were transfected with Lipofectamine 2000 at 3.3 µl of each siRNA (Dharmacon) or 9.9 µl of control or Qiagen siRNA. Infections with 5 PFU per cell were performed 24 h post-transfection and supernatants collected 48 h postinfection for quantification of CCL22 by ELISA. Alternatively, cells were lysed at the indicated time after transfection for reporter assay analysis or harvested directly into Laemmli's buffer. The levels of TRAF6 and TRAF2 were measured by Western blotting with anti-TRAF6 or anti-TRAF2 antibodies (Abcam), respectively. For Western blotting, proteins were detected by enhanced chemiluminescence using horseradish peroxidase conjugated sheep anti-mouse or donkey anti-rabbit IgG (Cell Signalling). UL144 levels in U373 cells after siRNA transfection were measured by RT-PCR. Total RNA was isolated using the RNeasy isolation system (Qiagen). After removal of the residual genomic DNA with DNase (Promega), first-strand cDNA was produced using the Promega RT System. PCR was performed using kit primers for GAPDH and 5'-GGCAGTATGTATCGG-3' and 5'-CCGCTTTTTCCTGTT-3' for Toledo UL144 and 5'-TCAGTGTTGTCCCCCA-3' and 5'-CGTTGCTTGTATGTT-3' for TB40E UL144 as predicted by 'Primer 3' (<http://frodo.wi.mit.edu/cgi-bin/primer3>).

Immunofluorescence and FRET

Cells were fixed and permeabilized (4% formaldehyde in PBS for 30 min RT, followed by 5 min RT of 25, 50, 75 and 100% ethanol consecutively). Cells were then rehydrated in decreasing concentrations of ethanol before washing in PBS. For GFP expressing cells, TO-PRO3 (Molecular Probes) was added. Infected cells were detected using Custom HCMV, FITC-conjugated antibody w/o Evans Blue (Chemicon International) specific for IE72 immediate early CMV protein, T7-tagged proteins using anti-T7-FITC conjugated goat polyclonal antibody (Abcam). T7 was also detected indirectly with anti-T7 monoclonal primary antibody (Novagen) followed by donkey anti-mouse TRITC (Sigma). TRAF6 and p65 were probed using primary antibodies (Abcam and Santa Cruz, respectively) followed by goat anti-rabbit conjugated to TRITC (Sigma) and concurrent DRAC5 (molecular probes) staining. Coverslips were mounted in Citifluor and examined using Nikon microscope and Image Pro 4.1 software or confocal microscope (LEICA DM IRBE) with TCS NT/SP series confocal software. The images were detected using excitation wavelengths of 488, 568 and 633 nm. FITC, TRITC and TO-PRO3/DRAC-5 were visualized in a sequential manner (one section, four accumulations) and saved as 'pre-bleach' scanner images. The area of interest for bleaching was selected with the 'zoom' tool. The TRITC stain was bleached by

scanning with the Krypton laser for 1 min at the maximum setting. Excitation was at 568 nm and emission at either 550–560 (FITC) or 600–650 nm (TRITC). Images were collected and saved as 'postbleach' scanner files for subsequent analysis with Image J software (<http://rsb.info.nih.gov>). For colocalization, the RG2B plugin was used with standard parameters. In FRET analysis, areas of interest (FITC stain) were outlined and measured with the software for pre- and postbleach images. The percentage of FRET efficiency was determined using the following equation: ((post-FRET–pre-FRET)/post-FRET) × 100 = % FRET efficiency. The efficiency of bleaching was determined in the same way but using the TRITC bleached areas (Poole *et al*, 2005).

Infections and inductions

HFF, U373 cells or 293-T cells were transfected and, where stated, super-infected with 5 PFU/cell of the indicated virus. To monitor the extent of virus replication, cells infected with IE72-GFP-Towne were analysed by FACS. Cells infected with TB40E were fixed 48 h postinfection in 70% ethanol at –20°C for 30 min and IE gene expression was assessed by GFP expression (Towne) or IE gene expression using FITC-conjugated mouse anti-IE72/IE86 antibody (Chemicon) or a mouse IgG isotype matched control antibody. Cells were analysed using FACS sort flow cytometer (Becton Dickinson, San Jose, CA) and FCS Express software (DeNovo Software, Ontario, Canada). For induction by synthetic dsRNA, poly(I)-poly(C) (Amersham Biosciences) was either added directly to the cells at a final concentration of 100 µg/ml or was transfected into cells using Lipofectamine 2000 (Invitrogen) under conditions specified by the manufacturer. Human recombinant TNF and type I human IFN-α2a (RND systems) were added to the cells at 10 ng/ml and 1000 IU/ml, respectively. Luciferase activity was analysed from cellular extracts and β-galactosidase activity was determined as a control of transfection.

Cytokine array and CCL22-specific ELISA

HFF or U373 cells were mock-infected or infected with HCMV Towne or TB40E. At 48 h postinfection, supernatants were harvested and assayed by RayBio[®] Human Cytokine Antibody Array C Series 100 Combination of Array VI and VII, following the manufacturer's instructions (RND systems). The CCL22 ELISA was carried out on supernatants from cells infected with HCMV TB40E, Toledo, Towne or AD169, or transfected with UL144 or siRNA as indicated and analysed in accordance with the manufacturer's protocol (RND systems).

Biomolecular interaction analysis using SPR

Approximately 800 response units of purified baculovirus-produced his-UL144 were immobilized by amine-coupling chemistry onto a CM-5 BIAcore chip with a BIAcoreX biosensor. During the association phase, human recombinant TNFSF-1, 2, 4, 5, 6, 8, 9, 10, 11, 12, 13, 13b, 14, 15 and 18 (RND systems) was diluted in running buffer HBS-EP (10 mM HEPES, pH 7.4/150 mM NaCl/3 mM EDTA/0.005% polysorbate 20 (vol/vol)) and applied individually to the sensor chip (30 µl/min). The UL144 sensor chip was regenerated with 10 mM acetate, pH 2.0. The data were analysed using the BIAevaluation 3.0 software.

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